

DERIVATIVES, CONJUGATES, AND ANTIBODIES FOR DETECTING ECSTASY-CLASS ANALYTES

Related Applications

[0001] This application is a continuation-in-part of U.S. Patent Application No. 10/087,612 filed March 1, 2002. Co-pending and commonly assigned U.S. Patent Application No. 10/087,469 was also filed March 1, 2002, and is incorporated herein by reference in its entirety.

Field of the Invention

[0002] The present invention relates to immunoassays, and more particularly, to immunoassays for derivatives of amphetamine, especially ecstasy-class drugs.

Background of the Invention

[0003] The use and abuse of a class of illicit designer drugs known commonly as "ecstasy-class drugs" have increased significantly in recent years. These compounds, which are derivatives of amphetamine distinguished by having a fused methylenedioxy phenyl ring system, include MDA (3,4-methylenedioxyamphetamine), MDMA, also known as "Ecstasy" (3,4-methylenedioxy-N-methylamphetamine), MDEA, also known as "Eve" (3,4-methylenedioxy-N-ethylamphetamine), BDB (3,4-methylenedioxyphenyl-2-butanamine), and MBDB (3,4-methylenedioxyphenyl-N-methylbutanamine).

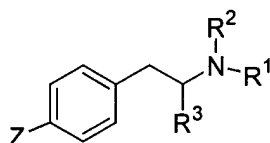
[0004] Heretofore, methods for the detection of ecstasy-class drugs have primarily involved immunoassays originally developed for the detection of amphetamine and/or methamphetamine. The detection of an ecstasy-class drug by such assays relies on the limited cross-reactivities that may coincidentally exist between the ecstasy-class drug and the amphetamine and/or methamphetamine antibodies. A positive result obtained by such an assay may still not indicate which particular substance or member of the methylenedioxy (MD) class of derivatives is present in a sample.

[0005] In general, amphetamine and methamphetamine immunoassays are relatively insensitive to, and non-specific for, ecstasy-class drugs. Such assays show particularly limited recognition for the MDEA derivative.

[0006] The present invention is directed to remedying these and other problems relating to the use of conventional amphetamine and/or methamphetamine immunoassays for the detection of members of the methylenedioxy class, or ecstasy-class, drugs.

Summary of the Invention

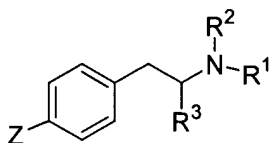
[0007] A compound embodying features of the present invention has the structure



wherein R^1 is an alkyl group comprising 2-6 carbon atoms; R^2 is selected from the group consisting of hydrogen, an alkyl group, and a protecting group; R^3 is an optionally substituted alkyl group; and Z is LXQ. Preferably R^1 is ethyl, propyl, or butyl, and more preferably, R^1 is ethyl. L comprises 1-15 carbon atoms and 0-6 heteroatoms. X is selected from the group consisting of O, CO, NR^4 , S, $C(=NH)O$, $NH(CO)$, $NH(CO)NH$, $NH(CS)$, $NH(CS)NH$, $O(CO)NH$, $NH(C=NH)$, and maleimidothioether, wherein R^4 is selected from the group consisting of hydrogen and an alkyl group. Q is selected from the group consisting of hydrogen, hydroxyl, leaving groups, macromolecular carriers, and labels.

[0008] A first antibody embodying features of the present invention is specific for an ecstasy drug.

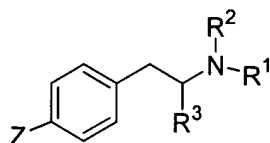
[0009] A second antibody embodying features of the present invention is generated in response to a compound having the structure



wherein R^1 is an alkyl group comprising 2-6 carbon atoms; R^2 is selected from the group consisting of hydrogen, an alkyl group, and a protecting group; R^3 is an optionally substituted alkyl group; and Z is LXQ. Preferably R^1 is ethyl, propyl, or butyl, and more preferably R^1 is ethyl. L comprises 1-15 carbon atoms and 0-6 heteroatoms. X is selected from the group consisting of O, CO, NR^4 , S, $C(=NH)O$, $NH(CO)$, $NH(CO)NH$, $NH(CS)$, $NH(CS)NH$, $O(CO)NH$, $NH(C=NH)$, and maleimidothioether, wherein R^4 is selected from the group consisting of hydrogen and an alkyl group. Q is selected from the group consisting of proteins, polypeptides, and polysaccharides.

[0010] A reagent kit embodying features of the present invention includes an antibody of the type described above.

[0011] A method for producing an antibody embodying features of the present invention includes inoculating a host with an immunogen comprising a structure



wherein R^1 is an alkyl group comprising 2-6 carbon atoms; R^2 is selected from the group consisting of hydrogen, an alkyl group, and a protecting group; R^3 is an optionally substituted alkyl group; and Z is LXQ. Preferably R^1 is ethyl, propyl, or butyl, and more preferably R^1 is ethyl. L comprises 1-15 carbon atoms and 0-6 heteroatoms. X is selected from the group consisting of O, CO, NR^4 , S, $C(=NH)O$, $NH(CO)$, $NH(CO)NH$, $NH(CS)$, $NH(CS)NH$, $O(CO)NH$, $NH(C=NH)$, and maleimidothioether, wherein R^4 is selected from the group consisting of hydrogen and an alkyl group. Q is a macromolecular carrier.

[0012] A method for detecting an analyte in a sample that embodies features of the present invention includes contacting the sample with an antibody of a type described above, binding the antibody to the analyte, and detecting, directly or indirectly, a complex formed by the antibody and the analyte.

Brief description of the drawings

[0013] Figure 1 shows a first representative scheme for synthesizing compounds and immunogens embodying features of the present invention.

[0014] Figure 2 shows a second representative scheme for synthesizing compounds and immunogens embodying features of the present invention.

[0015] Figure 3 shows a third representative scheme for synthesizing compounds embodying features of the present invention.

[0016] Figure 4 shows a calibration curve generated using KIMS immunoassay methodology and antibody MDMA 2.1.1, i.e., the antibody elicited from immunogen **12** in which T is KLH.

[0017] Figure 5 shows a calibration curve generated using KIMS immunoassay methodology and antibody NEAMP 48.2, i.e., an antibody elicited from immunogen **26** in which Q is KLH.

[0018] Figure 6 shows a calibration curve generated using KIMS immunoassay methodology and antibody NEAMP 62.1, i.e., an antibody elicited from immunogen **26** in which Q is KLH.

Detailed Description of the Invention

[0019] Compounds, e.g., haptens, intermediates, and immunogens useful in the production of antibodies specific for the MD class of amphetamine derivatives, antibodies specific for the MD class of amphetamine derivatives, reagent kits containing antibodies specific for the MD class of amphetamine derivatives, methods of producing antibodies specific for the MD class of amphetamine derivatives, and methods of detecting analytes including members of the MD class of amphetamine derivatives, i.e., ecstasy-class drugs, have been discovered and are described hereinbelow.

[0020] Throughout this description and in the appended claims, the following definitions are to be understood.

[0021] The term “immunogen” refers to any substance capable of eliciting an immune response in an organism.

[0022] The term “conjugate” refers to any substance formed from the joining together of two parts. Representative conjugates in accordance with the present invention include those formed by the joining together of a small molecule and a large molecule such as a protein. The term “conjugate” subsumes the term “immunogen.”

[0023] The term “hapten” refers to a portion of an immunogen that is typically low in molecular weight which does not by itself stimulate antibody development.

[0024] The phrase “activated hapten” refers to a hapten that has been provided with an available reaction site, for example, by the attachment of a linking group carrying a reactive moiety that can be used to connect the hapten to a carrier, immunogen, label, tracer, or other moiety.

[0025] The terms “linking group” and “linker” refer to a chemical moiety that is used to connect a hapten to a macromolecular carrier, label, tracer, or other moiety. The use of a linking group may or may not be advantageous or needed, depending on the specific hapten and carrier and desired specificity of the antibody. Suitable linkers include straight, branched, saturated, and unsaturated carbon chains which may incorporate one or more heteroatoms, that is, atoms other than carbon, e.g., oxygen, nitrogen, sulfur, etc., within the chain or substituted onto and/or at a terminus thereof.

[0026] The phrases “carrier” and “macromolecular carrier” refer to high molecular weight substances that can be coupled to haptens to form immunogens for antibody production and to form conjugates for use in an immunoassay. Suitable macromolecular carriers include, but are not limited to, proteins, glycoproteins, polymers, polysaccharides, polypeptides, and nucleic acids that are recognized as foreign and thereby elicit an immunologic response from a host.

[0027] The term “polypeptide” refers to any compound formed by the linkage of two or more amino acids via an amide bond. Representative polypeptides include polymers of α -amino acids in which the α -amino group of each non-terminal amino acid residue is linked to the α -carboxyl group of an adjacent residue in a linear chain. High molecular weight polypeptides are referred to as “proteins.”

[0028] The term “label” or “tracer” refers to an identifying tag that can be attached to a carrier substance or molecule and used to detect an analyte. A label may be attached to its carrier substance directly or indirectly by means of a linking or bridging moiety. Suitable labels include, but are not limited to, enzymes, e.g., β -galactosidase, peroxidase, etc., fluorescent compounds, e.g., rhodamine, fluorescein isothiocyanate or FITC, etc., luminescent compounds, e.g., dioxetanes, luciferin, etc., radioactive isotopes, e.g., ^{125}I , protein-binding partners, e.g., biotin, and the like.

[0029] The term “antibody” refers to a specific protein capable of binding an antigen or portion thereof. An antibody is produced in response to an immunogen which may have been introduced into a host, e.g., an animal or a human, by injection. The generic term “antibody” subsumes polyclonal antibodies, monoclonal antibodies, and antibody fragments.

[0030] The term “analyte” refers to any substance or group of substances, the presence or amount of which is to be determined. Representative ecstasy-class drug analytes include, but are not limited to, MDA, MDMA, MDEA, BDB, MBDB, and the like.

[0031] The term “derivative” refers to a chemical compound made from a parent compound by one or more chemical reactions.

[0032] The term “analyte analog” refers to any substance or group of substances, such as may be employed in a competitive immunoassay, which behaves similarly to an analyte with respect to binding affinity to an antibody. Representative analyte analogs include, but are not limited to, drugs, drug derivatives and isomers thereof, hormones, polypeptides, nucleotides, and the like. The analyte analog may also be the analyte itself.

[0033] The phrase “detecting an analyte” refers to any quantitative, semi-quantitative, or qualitative method as well as to all other methods for determining an analyte in general, and an ecstasy-class drug in particular. For example, a method that merely detects the presence or absence of an ecstasy-class drug in a sample lies within the scope of the present invention, as do methods that provide data as to the amount or concentration of the drug in the sample. The terms “detecting,” “determining,” “identifying,” and the like are used synonymously herein, and all lie within the scope of the present invention.

[0034] The phrase “reagent kit” refers to an assembly of materials and reagents that is used in performing an assay. The reagents can be provided in packaged combination in the same or in separate containers, depending on their cross-reactivities and stabilities, and in liquid or in lyophilized form. The amounts and proportions of reagents provided in the kit can be selected so as to provide optimum results for a particular application. A reagent kit embodying features of the present invention comprises antibodies specific for ecstasy-class drugs. The kit may further comprise analogs of the analyte and calibration and control materials. The reagents may remain in liquid form or may be lyophilized.

[0035] The phrase “calibration and control material” refers to any standard or reference material containing a known amount of an analyte. A sample suspected of containing an analyte and the corresponding calibration material are assayed under similar conditions. The concentration of analyte is calculated by comparing the results obtained for the unknown specimen with the results obtained for the standard. This is commonly done by constructing a calibration curve such as is shown in Figures 4 and 5.

[0036] The phrase “alkyl group” refers to any straight, branched, cyclic, acyclic, saturated, or unsaturated carbon chain. Representative alkyl groups include, but are not limited to, alkanes, alkenes, alkynes, cycloalkanes, cycloalkenes, cycloalkynes, aryls, and the like, and combinations thereof.

[0037] The phrase “optionally substituted” refers to the optional attachment of one or more substituents onto an alkyl group.

[0038] The phrase "leaving group" refers to any chemical moiety of a substrate that can be displaced by a reagent reacted therewith. Suitable leaving groups include, but are not limited to, halides, mesylates, tosylates, alkoxys, quaternary ammonium salts, and the like. Preferred leaving groups for use in accordance with the presently preferred embodiments are provided by activated esters, e.g., trifluoroethoxy esters, N-hydroxysuccinimide esters, p-nitrophenyl esters, pentafluorophenyl esters, imidazolyl esters, and N-hydroxybenzotriazolyl esters, whereby the oxygen-containing portion of the ester that is attached to the carbonyl carbon is displaced in the course of the reaction.

[0039] The phrase "protecting group" refers to any moiety that is attached to a reactive atom or center in order to alter its usual reactivity. Suitable protecting groups include, but are not limited to, those described in the treatise entitled "Protective Groups in Organic Synthesis", 3rd Edition, by Theodora W. Greene and Peter G. M. Wuts, John Wiley & Sons, Inc., New York, 1999, the entire content of which is incorporated herein by reference, except that in the event of any inconsistent disclosure or definition from the present application, the disclosure or definition herein shall be deemed to prevail. Various protecting groups for the nitrogen of amines are known in the art, e.g., *vide supra*, from amongst which trifluoroacetyl is a presently preferred nitrogen protecting group.

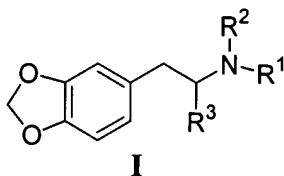
[0040] An antibody is said to be "specific" for an antigenic compound if it has the ability to discriminate that compound from other compounds.

[0041] "Cross-reactivity" refers to the reaction of an antibody with an antigen that was not used to induce that antibody.

[0042] An antibody is said to "preferentially bind" an antigenic compound if it exhibits greater binding of that compound than to other members of that class of compounds. For example, in Tables 2, 3, and 4, antibodies NEAMP 1.3, NEAMP 48.2, and NEAMP 62.1 bind preferentially to MDEA relative to other members in that class of compounds, i.e., methylenedioxy derivatives.

[0043] A compound embodying features of the present invention is useful as an intermediate, hapten, or immunogen in the production of antibodies specific for ecstasy-

class drugs. A first series of compounds embodying features of the present invention has the structure :



wherein R^1 is JMT, R^2 is selected from the group consisting of hydrogen, an alkyl group, and a protecting group, and R^3 is an optionally substituted alkyl group. J comprises 1-15 carbon atoms and 0-6 heteroatoms. M is selected from the group consisting of O, CO, NR^4 , S, $C(=NH)O$, $NH(CO)$, $NH(CO)NH$, $NH(CS)$, $NH(CS)NH$, $O(CO)NH$, $NH(C=NH)$, and maleimidothioether, wherein R^4 is selected from the group consisting of hydrogen and an alkyl group. T is selected from the group consisting of hydrogen, hydroxyl, leaving groups, macromolecular carriers, and labels. R^1 is not CH_2CN , $CH_2C=CH_2$, CHO , CH_2CH_2OH , $CH_2CH_2OCH_3$, or CH_2CCH when R^2 is hydrogen and when R^3 is methyl.

[0044] Preferably, the macromolecular carrier is selected from the group consisting of proteins, polypeptides, and polysaccharides. Preferred proteins include KLH (keyhole limpet hemocyanin), BSA (bovine serum albumin), and BTG (bovine thyroglobulin). Preferably, the alkyl groups comprise straight or branched chains and 1-15 carbon atoms, more preferably 1-11 carbon atoms, and still more preferably 1-9 carbon atoms.

[0045] In this first series of preferred embodiments, it is preferred that J comprises $(CH_2)_k$, wherein k is 1, 2, 3, 4, 5, or 6, and more preferably, k is 3. It is further preferred that M is CO. Preferably, R^2 is hydrogen, methyl, ethyl, *n*-propyl, or *n*-butyl, and more preferably, R^2 is hydrogen. Preferably, R^3 is hydrogen, methyl, ethyl, *n*-propyl, or *n*-butyl, and more preferably, R^3 is methyl. Preferably, T is selected from the group consisting of N-oxysuccinimide, hemocyanins, globulins, and albumins, and more preferably, T is selected from the group of proteins consisting of KLH, BSA, and BTG.

[0046] Figure 1 shows a representative scheme for synthesizing compounds and immunogens in accordance with this first series of preferred embodiments. It is to be

understood that in this representative synthetic scheme, the starting materials, reagents, individual synthetic transformations, and reaction conditions are purely illustrative and are not to be construed as limiting. Alternative synthetic preparations, including syntheses based on entirely different starting materials than the ones shown, can be developed without departing from the spirit and scope of the appended claims.

[0047] As shown in Figure 1, the synthesis begins with the ecstasy-class drug methylenedioxyamphetamine (MDA) **2**. The primary amino group of **2** is reacted with 4-bromo-butyric acid ethyl ester to give alkylation product **4**. The resultant secondary amino group of **4** is protected using a suitable amino protecting group. As shown in Figure 1, the amino group of **4** is trifluoroacetylated with trifluoroacetic anhydride (TFAA) to give the protected trifluoroacetylated derivative **6**. Hydrolysis of the ethyl ester moiety of **6** gives the carboxylic acid derivative **8**, which is esterified by reaction with N-hydroxysuccinimide (NHS) to give the activated ester derivative **10**. The activated ester derivative **10** is reacted with a macromolecular carrier moiety T, e.g., KLH, BTG, or BSA, deprotected, e.g., with potassium carbonate or at pH 13, and dialyzed to provide immunogen **12**.

[0048] Although the preferred moieties $(CH_2)_3$ and CO correspond to J and M, respectively, in compounds **6**, **8**, **10**, and **12** of Figure 1, it should be emphasized that the specific compounds shown in this synthesis are purely illustrative, and that the synthetic strategy outlined in Figure 1 can be modified to prepare compounds having substantially different chemical structures. For example, the alkylating agent 4-bromo-butyric acid ethyl ester shown in Figure 1 can be replaced with a reagent having more or less contiguous methylene units separating the leaving group, e.g., bromide, from the terminal functional group, e.g., the ethyl ester. Similarly, the carbon chain separating these termini can contain heteroatoms, substitution, unsaturation, or the like. Moreover, the functional group introduced through this alkylation step, i.e., the ethyl ester moiety of 4-bromo-butyric acid ethyl ester, can be replaced by a wide array of alternative moieties including, but not limited to, alcohols, protected alcohols, carboxylic acids, protected carboxylic acids, amines, e.g., primary, secondary, or tertiary, protected amines, thiols, protected thiols,

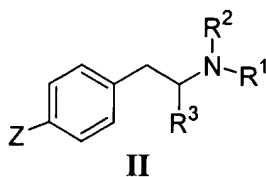
thioethers, amides, thioamides, imides, thioimides, nitriles, imines, hydrazones, maleimidothioethers, and the like, or by any functional group precursor to these moieties that can be converted thereto by one or more synthetic transformations, as is well established in the art.

[0049] Although the synthetic strategy outlined in Figure 1 introduces the J-M-T moiety through the alkylation of the amino group contained in MDA 2, it should be emphasized that this strategy of elaborating a methylenedioxy-phenyl ring system that already contains nitrogen is purely illustrative, and that numerous alternative strategies can be employed instead. For example, a methylenedioxy-phenyl ring system containing a leaving group in place of the amino group of MDA 2 can be reacted with an amino-containing nucleophile or with a nucleophile containing a precursor to an amino group, e.g., azide, cyanide, etc. Indeed, reacting an analog of MDA 2 containing a leaving group in place of the amino group with an amino analog of 4-bromo-butyric acid ethyl ester, that is, with $\text{NH}_2\text{-(CH}_2\text{)}_3\text{-CO}_2\text{Et}$, would also provide compound 6 via a different route. All manner of chemical transformations known in the art including, but not limited to, those described in treatises such as "Comprehensive Organic Transformations," 2nd Edition, by Richard C. Larock, Wiley-VCH, New York, 1999, and "March's Advanced Organic Chemistry," 5th Edition, by Michael B. Smith and Jerry March, John Wiley & Sons, Inc., 2001, and references cited therein, are contemplated for use in accordance with the presently preferred embodiments.

[0050] Transformations that may prove useful for modifications of the representative synthesis shown in Figure 1 include, but are by no means limited to, Fischer esterifications; preparation of other activated esters, e.g., with carbonyldiimidazole, dicyclohexylcarbodiimide, 2-chloropyridinium, 3-chloroisoxazolium, 2,2'-dipyridyl disulfide, 2-pyridyl thiochloroformate, and the like; oxidations, e.g., of alcohols, amines, thiols, thioethers, Baeyer-Villiger oxidation, etc.; reductions, e.g., reduction of nitro group, reductions of carbonyl groups, hydrogenation, etc.; protection of the amino group, e.g., carbamates amides, N-alkyl amines, N-aryl amines, imines, enamines, N-hetero atom derivatives, and the like and the corresponding deprotections; condensation reactions, e.g., aldol, Claisen, Knoevenagel, etc.; 1,4-addition reactions, e.g., Michael reaction, Corey-

Whitesides-House organocuprate coupling, etc.; 1,2-addition reactions, e.g., Grignard reactions, carbonyl reductions, etc.; reduction of nitriles; deprotection of alcohols; deprotection of carboxylic acids; deprotection of ketones; deprotection of aldehydes; reduction of azides; reductions of imines; and the like.

[0051] A second series of compounds embodying features of the present invention has the structure:



wherein R^1 is an alkyl group comprising 2-6 carbon atoms, R^2 is selected from the group consisting of hydrogen, an alkyl group, and a protecting group, R^3 is an optionally substituted alkyl group, and Z is L-X-Q. L comprises 1-15 carbon atoms and 0-6 heteroatoms. X is selected from the group consisting of O, CO, NR^4 , S, $C(=NH)O$, $NH(CO)$, $NH(CO)NH$, $NH(CS)$, $NH(CS)NH$, $O(CO)NH$, $NH(C=NH)$, and maleimidothioether, wherein R^4 is selected from the group consisting of hydrogen and alkyl groups. Q is selected from the group consisting of hydrogen, hydroxyl, leaving groups, macromolecular carriers, and labels.

[0052] Preferably, the macromolecular carrier is selected from the group consisting of proteins, polypeptides, and polysaccharides. Preferred proteins include KLH (keyhole limpet hemocyanin), BSA (bovine serum albumin), and BTG (bovine thyroglobulin). Preferably, the alkyl groups comprise straight or branched chains and 1-15 carbon atoms, more preferably 1-11 carbon atoms, and still more preferably 1-9 carbon atoms.

[0053] In this second series of preferred embodiments, the connectivity of carbon atoms and optional heteroatoms comprising L is unrestricted and may include straight, branched, cyclic, and acyclic systems. It is preferred that L comprise $(CH_2)_j$, wherein j is 1, 2, 3, 4, 5, or 6, and more preferably, j is 3. It is further preferred that X is CO. Preferably, R^1 is ethyl, *n*-propyl, or *n*-butyl, and more preferably, R^1 is ethyl. Preferably, R^2 is hydrogen or a

protecting group, and more preferably, R^2 is a protecting group such as the trifluoroacetyl group. Preferably, R^3 is hydrogen, methyl, ethyl, *n*-propyl, or *n*-butyl, and more preferably, R^3 is methyl. Preferably, Q is selected from the group consisting of hydroxy, N-oxy succinimide, hemocyanins, globulins, and albumins, and more preferably, Q is selected from the group of proteins consisting of KLH, BSA, and BTG.

[0054] Figure 2 shows a representative scheme for synthesizing compounds and immunogens in accordance with this second series of preferred embodiments. It is to be understood that in this representative synthetic scheme, the starting materials, reagents, individual synthetic transformations, and reaction conditions are purely illustrative and are not to be construed as limiting. Alternative synthetic preparations, including syntheses based on entirely different starting materials than the ones shown, can be developed without departing from the spirit and scope of the appended claims.

[0055] As shown in Figure 2, the synthesis begins with 1-methyl-2-phenyl-ethylamine **14**. The amino group of **14** is alkylated with ethyl bromide to give the N-ethylamine derivative **16**. The amino group of **16** is protected using a suitable amino protecting group. As shown in Figure 2, the amino group of **16** is trifluoroacetylated with trifluoroacetic anhydride (TFAA). Trifluoroacetylated derivative **18** is reacted with succinic anhydride in a Friedel-Crafts-type reaction to give the carboxylic acid derivative **20**. Reduction of the benzyl carbonyl group of the carboxylic acid derivative **20** gives the reduction product **22**, which is esterified by reaction with N-hydroxysuccinimide (NHS) to give the activated ester derivative **24**. The activated ester derivative **24** is reacted with a macromolecular carrier moiety Q, e.g., KLH, BSA, or BTG, the nitrogen deprotected under basic conditions and dialyzed to provide the immunogen **26**.

[0056] Alternatively, as shown in Figure 3, the activated ester derivative **24** can be further elaborated, for example, by reaction with 4-aminomethylbenzoic acid to give the benzoic acid derivative **32**. The benzoic acid derivative **32** and the activated ester derivative **34** obtained from **32** by reaction with N-hydroxysuccinimide are useful intermediates in the synthesis of a wide array of conjugates, labels, and the like in accordance with the present invention. The elaboration strategy outlined in Figure 3, i.e.,

introduction of the aminobenzoate moiety, can be easily adapted for use with methylenedioxy compounds of the type shown in Figure 1, e.g., by reacting the activated ester derivative **10** with 4-aminomethyl-benzoic acid.

[0057] Although the preferred moieties $(\text{CH}_2)_3$ and CO correspond to L and X, respectively, in compounds **22**, **24**, and **26** of Figure 2, it should be emphasized that the specific compounds shown in this synthesis are purely illustrative, and that the synthetic strategy outlined in Figure 2 can be modified to prepare compounds having substantially different chemical structures. For example, the succinic anhydride shown in Figure 2 can be replaced with a cyclic anhydride having more or fewer ring carbon atoms and/or ring heteroatoms, which themselves can be substituted, contain unsaturation, or the like. Moreover, there is no necessity to employ a cyclic anhydride as the Friedel-Crafts acylating agent. Acyclic reagents, e.g., acyl halides, carboxylic acids, ketones, etc., can also be employed. Furthermore, there is no necessity to employ a Friedel-Crafts acylation reaction to elaborate the structure of the phenyl ring as shown in Figure 2. A multitude of alternative electrophilic aromatic substitutions can also be employed including, but not limited to, Friedel-Crafts alkylation, halogenation, nitration, sulfonation, *ipso* substitution, and the like. Similarly, the functional group introduced through the Friedel-Crafts acylation step, i.e., the terminal carboxylic acid moiety shown in compounds **20** and **22**, can be replaced by or converted to a wide array of alternative moieties including, but not limited to, alcohols, protected alcohols, protected carboxylic acids, amines, e.g., primary, secondary, or tertiary, protected amines, thiols, protected thiols, thioethers, amides, thioamides, imides, thioimides, nitriles, imines, hydrazones, maleimidothioethers, and the like, or by any functional group precursor to these moieties that can be converted thereto by one or more synthetic transformations, as is well established in the art.

[0058] Although the synthetic strategy outlined in Figure 2 introduces the L-X-Q moiety through the acylation of the phenyl ring of the trifluoroacetylated derivative **18**, it should be emphasized that this strategy of elaborating a pre-existing phenyl ring by an electrophilic substitution is purely illustrative and that numerous alternative strategies could have been employed instead. For example, a phenyl ring substituted with a halogen,

e.g., Cl, Br, or I, at the position *para* to the amino-containing side chain can be converted to an organometallic reagent, e.g., a Grignard, an organolithium, an organostannane, an organoborane, an organocuprate, or the like and reacted with an electrophilic reagent, e.g., a ketone, an aldehyde, an acid halide, a haloalkane, etc., to form a carbon-carbon bond using procedures well-known in the art. Alternatively, a phenyl ring substituted with an appropriate leaving group, e.g., Cl, Br, I, alkoxy, etc., at the position *para* to the amino-containing side chain can be subjected to a nucleophilic aromatic substitution reaction using procedures well-known in the art. Furthermore, the substitution pattern of the phenyl ring could be developed on an entirely saturated or partially unsaturated cyclohexane ring system or precursor thereto, which is aromatized using reagents well-known in the art including, but not limited to, hydrogenation catalysts, e.g., Pt, Pd, Ni, etc., S and Se, quinines, and the like.

[0059] As noted above in reference to the synthetic scheme shown in Figure 1, all manner of chemical transformations known in the art are contemplated for use in accordance with the presently preferred embodiments. Transformations that may prove useful for modifications of the representative synthesis shown in Figure 2 include but are by no means limited to the ones identified above in reference to the synthetic scheme of Figure 1 as well as Wolff-Kishner reduction, Clemmensen reduction, the reduction of hydrazones, e.g., using LiAlH_4 , NaBH_4 , NaBH_3CN , or the like, and the like.

[0060] A first antibody embodying features of the present invention is specific and preferential for an ecstasy-class drug. Preferably, the ecstasy-class drug is selected from the group consisting of MDA, MDMA, MDEA, BDB, MBDB, and combinations thereof.

[0061] A second antibody embodying features of the present invention binds preferentially to MDEA and N-ethylamphetamine (NEAMP) with greater than 1% cross-reactivity to *d*-methamphetamine.

[0062] A third antibody embodying features of the present invention binds preferentially to MDEA with greater than 40% cross-reactivity to BDB and with greater than 1% cross-reactivity to *d*-amphetamine.

[0063] A fourth antibody embodying features of the present invention is generated in response to a compound having structure **I** or **II** shown and described above wherein Q is a macromolecular carrier.

[0064] Immunogens from the above-mentioned first series of preferred embodiments, that is, the series of compounds comprising a fused methylenedioxy-phenyl ring system, e.g., Figure 1, are useful for producing antibodies specific for ecstasy-class drugs including but not limited to MDA, MDMA, MDEA, BDB, MBDB, and combinations thereof. Table 1 shows cross-reactivity data for an antibody specific for ecstasy-class drugs, in particular for MDMA 2.1.1, which is an antibody generated in response to immunogen **12** of Figure 1 wherein T is KLH. The antibodies used in developing this data were produced through a classical immunization protocol of the type well established in the art. In Table 1, the abbreviation *d*-AMP represents *d*-amphetamine, the abbreviation *d*-MAMP represents *d*-methamphetamine, the abbreviation *l*-AMP represents *l*-amphetamine, the abbreviation *l*-MAMP represents *l*-methamphetamine, and the abbreviation MDPA represents 3,4-methylenedioxy-N-propylamphetamine.

[0065] Antibodies elicited by the immunogen **12**, e.g., wherein T is KLH, show good response and specificity to ecstasy-class drugs. Furthermore, these antibodies show little or no cross-reactivity to related drugs. By determining the drug concentration that results in a 50% reduction in binding (ED_{50}) of a standard, methamphetamine, dividing by the ED_{50} of each other drug, and then multiplying the result by 100, the percent cross-reactivity was calculated.

[0066] Percent cross-reactivity can also be determined in an immunoassay that employs antibodies and conjugates of this invention. A standard curve can be established using known amounts of the target analytes MDMA or MDEA. Known amounts of various amphetamine-related drugs can be analyzed as samples in the immunoassay, and apparent concentrations of these drugs can be generated from a standard curve. The apparent concentration of the drug divided by the actual concentration and multiplied by 100 equals the percent cross-reactivity of the drug. Such an immunoassay based on KIMS (kinetic interaction of microparticles in solution) is described in Example 20.

Table 1. Cross-reactivity of MDMA 2.1.1 with various drugs

Drug	% Cross-reactivity
MDMA	100
MDEA	204
MDA	60.6
MBDB	26.1
BDB	20.5
MDPA	365
<i>d</i> -AMP	0
<i>d</i> -MAMP	0.65
<i>l</i> -AMP	0
<i>l</i> -MAMP	0
sesamin	0
phentermine	0
tyramine	0
pseudoephedrine	0
ephedrine	0
phenylpropanolamine	0
norepinephrine	0
adrenaline	0
ranitidine	0

[0067] Immunogens from the above-mentioned second series of preferred embodiments, that is, the series of compounds lacking a fused methylenedioxy-phenyl ring system, are useful for producing antibodies for ecstasy-class drugs including but not limited to MDA, MDMA, MDEA, BDB, MBDB, and combinations thereof. Antibodies produced in response to N-ethyl-substituted immunogens from this second series, i.e., R¹ in structure **II** is ethyl, show particularly high recognition for the ecstasy-class drug MDEA, which is generally poorly detected by conventional amphetamine and methamphetamine

immunoassays. An antibody thus produced can be used either as a booster antibody to increase detection in an existing amphetamine or methamphetamine assay or as a separate antibody for MDEA in immunoassays for MD-class drugs.

[0068] Tables 2, 3, and 4 show cross-reactivity data for the antibodies NEAMP 1.3, NEAMP 48.2, and NEAMP 62.1, which were generated in response to immunogen 26 of Figure 2 wherein Q is KLH. By the procedure described above, using MDEA as a standard, the percent cross-reactivities shown in Tables 2, 3, and 4 were calculated. Table 2 shows cross-reactivity of NEAMP 1.3 generated in an ELISA assay, and Tables 3 and 4 show cross-reactivity of NEAMP 48.2 and NEAMP 62.1, respectively, in a KIMS immunoassay (ONLINE DAT II, Roche Diagnostics). The antibodies used in developing this data were produced using a classical immunization protocol.

Table 2. Cross-reactivity of NEAMP 1.3 with various drugs generated in an ELISA assay

Drug	% Cross-reactivity
<i>d</i> -methamphetamine	19.7
<i>d</i> -amphetamine	6.4
<i>l</i> -amphetamine	6.6
MDMA	22.5
MDEA	100
MBDB	3.9
phendimetrazine	0.12
<i>d</i> -pseudoephedrine	0.4
<i>l</i> -ephedrine	1.3
ranitidine	0.04

Table 3. Cross-reactivity of NEAMP 48.2 with various drugs
generated in a KIMS assay

Drug	% Cross-reactivity
<i>d</i> -methamphetamine	8
<i>l</i> -methamphetamine	0.5
<i>d</i> -amphetamine	0
<i>d</i> -N-ethylamphetamine	130
MDMA	5
MDEA	100
MBDB	6
BDB	1
phendimetrazine	0.04
<i>d</i> -pseudoephedrine	0.06
<i>l</i> -ephedrine	0

Table 4. Cross-reactivity of NEAMP 62.1 with various drugs
generated in a KIMS assay

Drug	% Cross-reactivity
<i>d</i> -methamphetamine	0.5
<i>l</i> -methamphetamine	0.2
<i>d</i> -amphetamine	1.1
<i>d</i> -N-ethylamphetamine	11
MDMA	4.0
MDEA	100
MBDB	13
BDB	46
phendimetrazine	0.1
<i>d</i> -pseudoephedrine	0.1
<i>l</i> -ephedrine	0.0

[0069] NEAMP 1.3 antibody, elicited by the N-ethyl substituted immunogen **26**, e.g., wherein Q is KLH, shows good response, specificity, and preference for ecstasy-class drugs in general and to MDEA in particular.

[0070] NEAMP 48.2 antibody, elicited by the N-ethyl substituted immunogen **26**, e.g., wherein Q is KLH, shows good response and preference for MDEA and N-ethylamphetamine, as shown by quantitative KIMS immunoassay in Table 3. The antibody also has greater than 5% cross-reactivity to d-methamphetamine. The murine hybridoma cell line NEAMP 48.2 was deposited with the American Type Culture Collection (ATCC, Manassas, VA) on June 26, 2003 and assigned ATCC designation PTA-5295.

[0071] NEAMP 62.1 antibody, elicited by the N-ethyl substituted immunogen **26**, e.g., wherein Q is KLH, shows good response and preference for MDEA and good response to BDB, as shown by quantitative KIMS immunoassay data in Table 4. The antibody also has greater than 1% cross-reactivity to *d*-amphetamine. The murine hybridoma cell line NEAMP 62.1 was deposited with the American Type Culture Collection on June 26, 2003 and assigned ATCC designation PTA-5294.

[0072] A reagent kit embodying features of the present invention comprises an antibody embodying features of the present invention. A representative reagent kit may comprise an antibody specific for an ecstasy-class drug, a complex comprising an analog of an ecstasy-class drug or a derivative thereof coupled to a labeling moiety, and may optionally also comprise one or more calibrators comprising a known amount of an ecstasy-class drug or a related standard.

[0073] Antibodies embodying features of the present invention can be included in a kit, container, pack, or dispenser together with instructions for their utilization. When the antibodies are supplied in a kit, the different components of the immunoassay may be packaged in separate containers and admixed prior to use. Such packaging of the components separately may permit long-term storage without substantially diminishing the functioning of the active components. Furthermore, reagents can be packaged under inert

environments, e.g., under a positive pressure of nitrogen gas, argon gas, or the like, which is especially preferred for reagents that are sensitive to air and/or moisture.

[0074] Reagents included in kits embodying features of the present invention can be supplied in all manner of containers such that the activities of the different components are substantially preserved while the components themselves are not substantially adsorbed or altered by the materials of the container. Suitable containers include, but are not limited to, ampules, bottles, test tubes, vials, flasks, syringes, envelopes, e.g., foil-lined, and the like. The containers may be comprised of any suitable material including but not limited to glass, organic polymers, e.g., polycarbonate, polystyrene, polyethylene, etc., ceramic, metal, e.g., aluminum, metal alloys, e.g., steel, cork, and the like. In addition, the containers may comprise one or more sterile access ports, e.g., for access via a needle, such as may be provided by a septum. Preferred materials for septa include rubber and polytetrafluoroethylene of the type sold under the trade name TEFLON by DuPont (Wilmington, DE). In addition, the containers may comprise two or more compartments separated by partitions or membranes that can be removed to allow mixing of the components.

[0075] Reagent kits embodying features of the present invention may also be supplied with instructional materials. Instructions may be printed, e.g., on paper and/or supplied in an electronically-readable medium, e.g., floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, audio tape, etc. Alternatively, instructions may be provided by directing a user to an internet web site, e.g., specified by the manufacturer or distributor of the kit and/or via electronic mail.

[0076] As noted above, reagent kits embodying features of the present invention may comprise calibration and/or control materials which comprise a known amount of the analyte to be measured. The concentration of an analyte can be calculated by comparing results obtained for a sample with results obtained for a standard. A calibration curve can be constructed and used for relating the sets of results and for determining the concentration of an analyte in a sample. Figures 4 and 5 show calibration curves generated using a Roche/HITACHI analyzer (Roche Diagnostics) using modified Roche ONLINE

DAT II formats and reagents, i.e., KIMS, and antibodies NEAMP 48.2 and NEAMP 62.1, respectively, i.e., the antibodies elicited from immunogen **26** in which Q is KLH.

[0077] Methods of detecting an analyte that embody features of the present invention comprise contacting a sample with an antibody embodying features of the present invention, binding the antibody to the analyte, and detecting a complex formed by the antibody and the analyte.

[0078] Any sample that is suspected of containing an analyte, e.g., an ecstasy-class drug, can be analyzed in accordance with the methods of the presently preferred embodiments. The sample can be pretreated if desired and can be prepared in any convenient medium that does not interfere with the assay. Preferably, the sample comprises an aqueous medium such as a body fluid from a host. Representative bodily fluids include, but are not limited to, urine, whole blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, and the like, and combinations thereof. Preferably, the bodily fluid comprises plasma, serum, or urine.

[0079] It is to be understood that all manner of immunoassays employing antibodies are contemplated for use in accordance with the presently preferred embodiments, including assays in which antibodies are bound to solid phases and assays in which antibodies are in liquid media. Methods of immunoassay that can be used to detect analytes using antibodies embodying features of the present invention include, but are not limited to, competitive (reagent limited) assays wherein labeled analyte (analyte analog) and analyte in a sample compete for antibodies; single-site immunometric assays wherein the antibody is labeled; two-site immunometric (reagent excess) assays wherein a capture antibody, i.e., an antibody attached to a solid phase, binds a first epitope of an antigen, and wherein a detecting antibody, i.e., a labeled antibody, binds to the antigen-capture antibody complex; and the like.

[0080] In this invention, one of the assay methods used was the Roche ONLINE DAT II methodology, an immunoassay based on kinetic interaction of microparticles in solution (KIMS). In this assay, antibodies are coated onto polystyrene particles, and one of the

compounds described in this invention is conjugated to a high molecular weight carrier. In the absence of the ecstasy-class compound, the antibody will bind to the conjugate, forcing particle aggregation and thus producing a change in the absorbance of the reagent. In the presence of the ecstasy-class compound, binding of the antibody to the conjugate and the corresponding change in the absorbance will be prevented. The amount of absorbance generated by aggregating particles can be correlated with the amount of the free drug present in the sample.

[0081] Procedures for performing various types of immunoassays are well established in the art and are set forth in numerous treatises and publications including “The Immunoassay Handbook,” 2nd Edition, edited by David Wild, Nature Publishing Group, 2000, the entire content of which is incorporated herein by reference, except that in the event of any disclosure or definition inconsistent with the present application, the disclosure or definition herein shall be deemed to prevail.

[0082] Methods of producing antibodies embodying features of the present invention comprise inoculating a host with an immunogen embodying features of the present invention. Suitable hosts include, but are not limited to, mice, rats, hamsters, guinea pigs, rabbits, chickens, donkeys, horses, monkeys, chimpanzees, orangutans, gorillas, humans, and any species capable of mounting a mature immune response. The immunization procedures used are well established in the art and are set forth in numerous treatises and publications including “The Immunoassay Handbook,” 2nd Edition, cited above, and the references cited therein.

[0083] Preferably, an immunogen embodying features of the present invention is administered to a host subject, e.g., an animal or a human, in combination with an adjuvant. Suitable adjuvants include, but are not limited to, Freund’s adjuvant, powdered aluminum hydroxide (alum), aluminum hydroxide together with *Bordetella pertussis*, and monophosphoryl lipid A-synthetic trehalose dicorynomycolate (MPL-TDM).

[0084] Polyclonal antibodies can be raised in a mammalian host by one or more injections of an immunogen which can optionally be administered together with an

adjuvant. Typically, an immunogen or a combination of an immunogen and an adjuvant is injected into a mammalian host by one or multiple subcutaneous or intraperitoneal injections. Preferably, the immunization program is carried out over at least one week, and more preferably, over two or more weeks. Polyclonal antibodies produced in this manner can be isolated and purified utilizing methods well known in the art.

[0085] Monoclonal antibodies can be produced by the well-established hybridoma methods of Köhler and Milstein, e.g., *Nature* 256, pp. 495-497, 1975. Hybridoma methods typically involve immunizing a host or lymphocytes from a host, harvesting the monoclonal antibody secreting or having the potential to secrete lymphocytes, fusing the lymphocytes to immortalized cells, and selecting cells that secrete the desired monoclonal antibody.

[0086] A host can be immunized to elicit lymphocytes that produce or are capable of producing antibodies specific for an immunogen. Alternatively, the lymphocytes can be immunized *in vitro*. If human cells are desired, peripheral blood lymphocytes can be used, although spleen cells or lymphocytes from other mammalian sources are preferred.

[0087] The lymphocytes can be fused with an immortalized cell line to form hybridoma cells, a process which can be facilitated by the use of a fusing agent, e.g., polyethylene glycol. By way of illustration, mutant rodent, bovine, or human myeloma cells immortalized by transformation can be used. Substantially pure populations of hybridoma cells, as opposed to unfused immortalized cells, are preferred. Thus, following fusion, the cells can be grown in a suitable medium that inhibits the growth or survival of unfused, immortalized cells, for example, by using mutant myeloma cells that lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). In such an instance, hypoxanthine, aminopterin, and thymidine can be added to the medium (HAT medium) to prevent the growth of HGPRT-deficient cells while permitting hybridomas to grow.

[0088] Preferably, immortalized cells fuse efficiently, can be isolated from mixed populations by selection in a medium such as HAT, and support stable and high-level

expression of antibody following fusion. Preferred immortalized cell lines include myeloma cell lines available from the American Type Culture Collection, Manassas, VA.

[0089] Because hybridoma cells typically secrete antibody extracellularly, the culture media can be assayed for the presence of monoclonal antibodies specific for the MD-class of amphetamine derivatives. Immunoprecipitation or *in vitro* binding assays, for example, radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA), can be used to measure the binding specificity of monoclonal antibodies.

[0090] Monoclonal antibody-secreting hybridoma cells can be isolated as single clones by limiting dilution procedures and sub-cultured. Suitable culture media include, but are not limited to, Dulbecco's Modified Eagle's Medium, RPMI-1640, and polypeptide-free, polypeptide-reduced, or serum-free media, e.g., Ultra DOMA PF or HL-1, available from Biowhittaker, Walkersville, MD. Alternatively, the hybridoma cells can be grown *in vivo* as ascites.

[0091] Monoclonal antibodies can be isolated and/or purified from a culture medium or ascites fluid by conventional immunoglobulin (Ig) purification procedures including, but not limited to, polypeptide A-SEPHAROSE, hydroxylapatite chromatography, gel electrophoresis, dialysis, ammonium sulfate precipitation, and affinity chromatography.

[0092] Monoclonal antibodies can also be produced by recombinant methods such as are described in U.S. Patent No. 4,166,452. DNA encoding monoclonal antibodies can be isolated and sequenced using conventional procedures, e.g., using oligonucleotide probes that specifically bind to murine heavy and light antibody chain genes, preferably to probe DNA isolated from monoclonal antibody hybridoma cell lines secreting antibodies specific for ecstasy-class drugs. The isolated DNA fragments can be sub-cloned into expression vectors that are then transfected into host cells, for example, simian COS-7 cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce Ig polypeptide, to express monoclonal antibodies. The isolated DNA fragments can be modified by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, as described in U.S. Patent No.

4,816,567 or by fusing the Ig coding sequence to all or a portion of the coding sequence for a non-Ig polypeptide. Such a non-Ig polypeptide can be substituted for the constant domains of an antibody or can be substituted for the variable domains of one antigen-combining site to create a chimeric bivalent antibody.

[0093] The following representative procedures for preparing immunogens embodying features of the present invention and for developing hybridomas to ecstasy-class drugs are provided solely by way of illustration and are not intended to limit the scope of the appended claims or their equivalents.

Examples

[0094] Chemical reagents were obtained from Aldrich Chemical Co., Milwaukee, WI, USA, unless otherwise stated. Solvents were obtained from either J.T. Baker or Fisher Scientific and were of ACS or HPLC grade or better unless otherwise stated. Methylene chloride (CH_2Cl_2) was dried by distillation over and from calcium hydride. Tetrahydrofuran (THF) was dried by distillation over and from sodium and benzophenone. Dry dimethylformamide (DMF) was obtained from Aldrich Chemical Co. in sealed SURESEAL bottles. Column chromatography was performed using E.M. Science flash-grade silica gel (Cat. No. 9385-9, Silica Gel 60, 230-400 mesh ASTM). Thin layer chromatography was performed using silica gel plates obtained from E.M. Science (Cat. No. 5715-7, 0.025 cm thickness). "KPi" refers to potassium phosphate buffer. Mixed solvents are expressed as volume for volume percentages, e.g., 10% MeOH- CHCl_3 or 10% MeOH in CHCl_3 refers to chloroform containing 10% of methanol by volume.

Example 1. Synthesis of MDA derivative 4

[0095] A suspension/solution of 700 mg of methylenedioxyamphetamine hydrobromide salt in methylene chloride (CH_2Cl_2) was shaken thoroughly with saturated aqueous sodium bicarbonate (NaHCO_3). The layers were separated and the aqueous layer extracted repeatedly with additional CH_2Cl_2 until only negligible organic material was being extracted. The combined organic layers were evaporated to dryness under reduced pressure

using a rotary evaporator (rotovap) and briefly dried further under high vacuum to give 408 mg of the free base of methylenedioxyamphetamine **2** as an oil.

[0096] To a solution of 400 mg of the free base **2** in 5 mL of dry dimethylformamide (DMF) was added 387 μ L (1.2 mol. equiv.) of ethyl 4-bromobutyrate (Fluka Chemical Co.) and the reaction stirred overnight at room temperature under argon. The reaction mixture was diluted with 20 mL of CH_2Cl_2 , stirred with 25 mL of saturated aqueous NaHCO_3 , the layers separated, the aqueous layer extracted with 50 mL CH_2Cl_2 followed by 50 mL ethyl acetate (EtOAc), the organic extracts combined, dried over sodium sulfate (Na_2SO_4), evaporated under reduced pressure (rotovap), and the residue dried under high vacuum (manifold) to give 520 mg of the product **4**, shown by ^1H -NMR to be about 90% pure. The material was used without further purification in the next step.

[0097] Material obtained from the extraction of a similar reaction after the aqueous quench indicated the presence of the product **4** as the HBr salt, together with small amounts of the disubstituted product, by ^1H -NMR. Silica gel chromatographic purification [1st column, 20% methanol (MeOH) in chloroform (CHCl_3) as eluent; 2nd column, EtOAc-MeOH-acetone-water (6:1:1:1) as eluent] gave clean product **4**. Mass spec M-H, 292.

Example 2. Synthesis of **6**

[0098] To a solution of 500 mg of crude **4** and 950 μ L (4 mol. equiv.) of triethylamine in dry CH_2Cl_2 under argon and cooled to about 0°C (ice bath) was added 289 μ L (1.2 mol. equiv.) of trifluoroacetic anhydride (TFAA). The reaction was allowed to warm up to room temperature while stirring overnight. The reaction was diluted to a volume of 50 mL with CH_2Cl_2 , washed with water (2 x 50 mL), saturated aqueous NaHCO_3 (2 x 50 mL), saturated aqueous sodium chloride (NaCl , 1 x 50 mL), dried over Na_2SO_4 , evaporated (rotovap) and dried under high vacuum to give about 730 mg of crude product. The material was chromatographed on silica gel, eluting with 30% EtOAc in hexanes, to give 449 mg of the product **6** as a pale colored liquid. Mass spec (M+H): Observed, 389.1449; Calc, 389.1450.

Example 3. Synthesis of 8

[0099] A solution of 445 mg of **6** in 2 mL of THF and 2 mL of 3 N perchloric acid was stirred at 50°C (oil bath) under argon for 4.5 hours. The reaction was poured into 75 mL of water, the mixture extracted with EtOAc (2 x 50 mL), the organic extracts washed with water, dried (Na₂SO₄), and evaporated (rotovap) to give 416 mg of crude product. The material was chromatographed on silica gel, eluting with 5% MeOH in CH₂Cl₂, the fractions containing product combined, evaporated (rotovap), and dried under high vacuum to give 320 mg of product **8** as a collapsing foam. Low resolution mass spec (M+H): Observed, 362.1. High resolution mass spec (M+Na): Observed, 384.1024; Calc, 384.1035.

Example 4. Synthesis of 10

[00100] A solution of 310 mg of **8** in 20 mL of dry CH₂Cl₂ under argon was treated with 296 mg (3 mol. equiv.) of N-hydroxysuccinimide followed by 329 mg (2 mol. equiv.) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, Sigma Chemical Co.) and stirred overnight at room temperature. The reaction was washed with water (1 x 20 mL), saturated aqueous NaHCO₃ (2 x 20 mL), saturated aqueous NaCl (1 x 20 mL), dried (Na₂SO₄), and evaporated (rotovap). The residue was chromatographed on silica gel, eluting with 30% EtOAc in hexanes, the product fractions combined and evaporated (rotovap). The residue was redissolved in dry CH₂Cl₂ and re-evaporated (6 times), then dried under high vacuum to give 280 mg of the NHS ester derivative **10** as a white/colorless collapsing foam. High res mass spec (M+H): Observed, 459.1381; Calc, 459.1379.

Example 5. Synthesis of MDMA immunogen 12 (T=KLH, 12a)

[00101] To a stirring solution of 220 mg of purified keyhole limpet hemocyanin (KLH) in 13 mL of 50 mM KPi, pH 7.5, cooled in an ice-water bath was added 4.33 mL of dimethylsulfoxide (DMSO) dropwise to give a solution of KLH in 25% DMSO-KPi. 1.58 mL, equivalent to about 20 mg protein, was withdrawn for use as the control. To the

remainder was added a solution of 26 mg of **10** (about 0.6 equiv. per lysine in KLH) dissolved in a total of 1.5 mL DMSO, giving a reaction of **10** with KLH in about 31% DMSO-KPi. The ice bath was removed and the reaction (stoppered flask) stirred overnight. The opalescent gray reaction was transferred to dialysis tubing (15,000 MW cut-off; SpectraPor 7) and dialyzed sequentially against 30% DMSO-KPi/room temperature (3 x 1.1 L), 15% DMSO-KPi/room temperature, then KPi (1 x 2.2 L/room temperature, greater than about 4°C; 5 x 2.2 L/about 4°C). All KPi was 50 mM KPi, pH 7.5. The control KLH was also transferred to dialysis tubing (15,000 MW cut-off; SpectraPor 7) and dialyzed separately against 30% DMSO-KPi, then placed in the same dialysis vessel with the immunogen when stepping down to 15% DMSO-KPi. 1 mL of the retentate was removed for determination of the extent of lysine modification. The remainder was dialyzed against 50 mM K₂CO₃ (4 x 2.2 L/room temperature/2 days) then against KPi (4 x 2.2 L/about 4°C). Deprotection of the amine was completed by redialysis against pH 13 buffer (50 mM K₂CO₃ basified with KOH to pH 13) at room temperature for about 7 days followed by dialysis back into 50 mM KPi pH 7.5 (3 changes) to give the MDMA immunogen **12** (T=KLH, **12a**) as an almost colorless, clear solution. Coomassie Blue protein assay (modified Bradford assay, Biorad Laboratories, Hercules, CA) gave 1.9 mg/mL protein. Trinitrobenzenesulfonic acid (TNBS) assay on the undeprotected immunogen (*vide supra*, after protein concentration determination via the Coomassie Blue assay) gave 38% modification of available lysines on KLH.

Example 6. Synthesis of the MDMA conjugate **12 (T=BSA, **12b**)**

[00102] To a stirring solution of 0.55 g of bovine serum albumin (BSA, Pentex Fraction V, Miles Inc., Kankakee, IL) in 11 mL of 50 mM KPi, pH 7.5, cooled in an ice-water bath was added 4.0 mL of DMSO dropwise. From the resulting solution of BSA in about 27% DMSO-KPi was withdrawn 1.36 mL containing about 0.05 g of BSA for use as the control if needed. To the remaining solution was added 8.2 mg (about 2.4 mol. equiv.) of **10** dissolved in a total of 0.6 mL of DMSO, resulting in a mixture of **10** and BSA in 30% DMSO-KPi. The ice-water bath was removed and the reaction allowed to stir overnight in a stoppered flask. The clear reaction was transferred to dialysis tubing (15,000 MW cut-

off, SpectraPor 7) and dialyzed sequentially against 30% DMSO-KPi/room temperature (1.1 L), 15% DMSO-KPi/room temperature (1.1 L), KPi/room temperature (1 x 1.1), then 50 mM K₂CO₃ (4 x 1.1 L/room temperature/2 days), then against KPi (4 x 2.2 L/about 4°C. All KPi was 50 mM KPi, pH 7.5. The control KLH was also transferred to dialysis tubing (15,000 MW cut-off, SpectraPor 7) and dialyzed separately against 30% DMSO-KPi, then placed in the same dialysis vessel with the immunogen when stepping down to 15% DMSO-KPi and carried forward alongside. Analysis of a portion of the retentate showed the protein concentration to be 18.9 mg/mL (Coomassie Blue protein assay) and the substitution by hapten to be about 1.6 (difference UV, against the BSA control). Deprotection of the amine was completed by redialysis against pH 13 buffer (50 mM K₂CO₃ basified with KOH to pH 13) at room temperature for about 4 days followed by dialysis back into 50 mM KPi pH 7.5 (4 changes) to give the MDMA conjugate **12** (T=BSA, **12b**) as a colorless clear solution. The protein concentration was determined by UV (OD₂₈₀ of conjugate taken to be approximately the same as OD₂₈₀ of parent BSA=0.6 at 1 mg/mL) to be approximately 1.9 mg/mL protein.

Example 7. Synthesis of N-ethylamphetamine 16

[00103] 5.0 g of *d*-amphetamine sulfate (Sigma Chemical Co., St. Louis, MO) was treated with 100 mL of CH₂Cl₂ and 30 mL of 1 N NaOH and stirred vigorously for 15 minutes. The layers were separated, and the aqueous portion was extracted with 25 mL of CH₂Cl₂. The organic portions were combined, dried over anhydrous Na₂SO₄, and concentrated at reduced pressure to give 3.66 g of *d*-amphetamine free base **14** as a clear oil. This was dissolved in 30 mL of anhydrous DMF, treated with 2.9 g of ethyl bromide, and stirred at room temperature for 3 days. The mixture was concentrated at reduced pressure to yield 6.6 g and used crude in the next step. The product contained some starting material and diethylated by-product, which is difficult to purify by column chromatography.

Example 8. Synthesis of 18

[00104] A solution of 6.6 g of crude N-ethylamphetamine in 75 mL of anhydrous CH₂Cl₂ was treated with 10 mL of triethylamine. The mixture was cooled with an ice bath, treated

with 4.3 mL of trifluoroacetic anhydride, and stirred at room temperature under argon overnight. The mixture was concentrated at reduced pressure. The residue was dissolved in 75 mL of EtOAc and washed with 3 x 25 mL of saturated NaHCO₃, 25 mL of H₂O, 25 mL of saturated brine, dried over anhydrous Na₂SO₄, and concentrated at reduced pressure. The residue was chromatographed on 300 g of silica gel using 30% EtOAc-hexane as eluent to yield 4.0 g of clear oil, which still contained some diethylated by-product from the previous step. This was rechromatographed on 250 g of silica gel using 5% EtOAc-hexane as eluent to yield 2.6 g of **18** as a clear oil.

Example 9. Synthesis of 20

[00105] A solution of 2.0 g of **18** in 50 mL of anhydrous CH₂Cl₂ under argon was treated with 1.2 g of succinic anhydride. The mixture was cooled with an ice bath, then treated with 4.0 g of AlCl₃ added portionwise. The reaction was stirred at 0 °C for 2 hours, then at room temperature overnight. The mixture was treated with 18 mL of 3 N HCl added slowly at first, then stirred vigorously for 30 minutes. The layers were separated, and the organic layer was washed with 25 mL of H₂O and 25 mL of saturated brine, dried over Na₂SO₄, and concentrated at reduced pressure to an amber oil. This was chromatographed on 150 g of silica gel using 3% MeOH-CH₂Cl₂ as eluent to yield 2.6 g of **20** as an amber oil.

Example 10. Synthesis of 22

[00106] A 500 mL Parr bottle was charged with 115 mg of 10% Pd/C followed by a solution of 600 mg of **20** in 30 mL of acetic acid and hydrogenated at 50 psi for 17 hours. The catalyst was filtered off through the filter agent sold under the tradename CELITE by Celite Corporation (available from Aldrich Chemical Company, Inc., Milwaukee, WI), and the filtrate was concentrated at reduced pressure. Residual acetic acid was driven off by evaporating 5 times with 25 mL of toluene. The toluene was driven off by evaporating 5 times with CH₂Cl₂ to yield 576 mg of **22** as an amber oil.

Example 11. Synthesis of 24

[00107] A solution of 576 mg of **22** in 25 mL of anhydrous CH_2Cl_2 under argon was treated with 260 mg of N-hydroxysuccinimide followed by 435 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl and stirred at room temperature overnight. The mixture was washed with 25 mL of 0.1 N HCl, 25 mL of H_2O , 2 x 25 mL of saturated NaHCO_3 , 25 mL of saturated brine, dried over Na_2SO_4 , and concentrated at reduced pressure to yield 735 mg of **24** as an amber oil.

Example 12. Synthesis of 32

[00108] A mixture of 108 mg of 4-(aminomethyl)benzoic acid in 5 mL of H_2O and 10 mL of distilled THF was treated with a solution of 315 mg of **24** in 10 mL of distilled THF followed by 1.2 mL of 1 N NaOH and stirred at room temperature for 1 hour. The pH of the reaction was 9. The THF was removed at reduced pressure, and the aqueous residue was diluted with 5 mL of H_2O and acidified to pH 6 with 6 N HCl. This was extracted with 2 x 15 mL of EtOAc. The EtOAc extracts were combined, dried over anhydrous Na_2SO_4 , and concentrated *in vacuo* to yield 290 mg of **32** as a white amorphous solid.

Example 13. Synthesis of 34

[00109] A solution of 270 mg of **32** in 10 mL of anhydrous CH_2Cl_2 under argon was treated with 85 mg of N-hydroxysuccinimide followed by 140 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl and stirred at room temperature overnight. The mixture was diluted with 10 mL of CH_2Cl_2 , washed with 10 mL of 0.1 N HCl, 10 mL of saturated brine, 2 x 10 mL of saturated NaHCO_3 , 10 mL of saturated brine, dried over Na_2SO_4 , and concentrated at reduced pressure to a white amorphous solid. This was chromatographed on 80 g of silica gel using EtOAc as eluent to yield 190 mg of **34** as a white amorphous solid.

Example 14. Synthesis of N-ethylamphetamine immunogen 26 (Q=KLH, 26a)

[00110] A solution of 342 mg of purified KLH in 10 mL of 50 mM KPi, pH 7.5, was cooled with an ice bath and treated with 4 mL of DMSO added dropwise. 1.7 mL was removed, which was used as a reference. This left 300 mg of KLH in solution. This was then treated with a solution of 50 mg of **24** in 1.0 mL of DMSO added dropwise. The reaction was stirred at room temperature overnight. The reaction and the reference sample were placed in separate 10,000 MW cut-off dialysis tubing (SpectraPor 7) and dialyzed in 1 liter of 33% DMSO-50 mM KPi, pH 7.5, at room temperature, 3 changes, at least 3 hours each, the last one going overnight. This was then dialyzed using a step-down gradient in 1 liter of 20% DMSO, 1 liter of 10% DMSO, 1 liter of 100% KPi, pH 7.5, at room temperature, at least 3 hours each. The bags were then placed in 1 liter of 50 mM K₂CO₃, pH 11.4, and dialyzed for 4 days at 40°C (changed once on day 2). This was then dialyzed in 1 liter of 50 mM KPi, pH 7.5, at 4°C, 6 changes at least 6 hours each. Coomassie Blue protein assay (modified Bradford assay, Biorad Chemical Co.) gave a protein concentration of 8.16 mg/mL. Trinitrobenzenesulfonic acid (TNBS) assay on a protected sample gave 41.4% of available lysines modified.

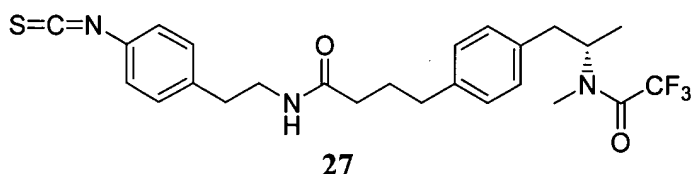
Example 15. Synthesis of N-ethylamphetamine conjugate 26 (Q=BSA, 26b)

[00111] A solution of 500 mL of bovine serum albumin (BSA, Cohn Fraction V modified powder, Intergen Company, Purchase, NY) in 8 mL of 50 mM KPi, pH 7.5, was cooled with an ice bath and treated with 11 mL of DMSO added slowly dropwise. This was then treated with a solution of 6.7 mg of **24** in 1 mL of DMSO added dropwise and stirred at room temperature overnight. The mixture was placed into 10,000 MW cut-off dialysis tubing (SpectraPor 7) and dialyzed in 1 liter of 60% DMSO-50 mM KPi, pH 7.5, at room temperature, 3 changes at least 3 hours each, the last one going overnight. This was then dialyzed using a step-down gradient in 1 liter of 40% DMSO, 1 liter of 20% DMSO, 1 liter of 10% DMSO, and 1 liter of 100% 50 mM KPi, pH 7.5, at room temperature, at least 3 hours each. This was then dialyzed in 1 liter of 50 mM K₂CO₃ (adjusted to pH 13 with KOH) for 4 days with 4 changes of buffer. This was then dialyzed in 1 liter of 50 mM KPi,

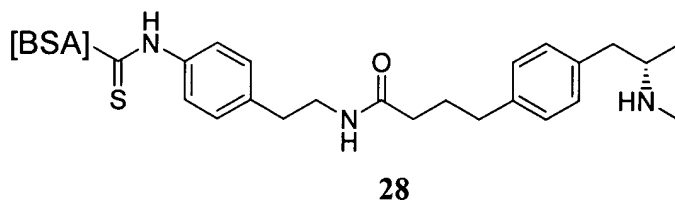
pH 7.5, at 4°C, 6 changes, at least 6 hours each. Coomassie Blue protein assay gave a protein concentration of 12.2 mg/mL.

Example 16. Synthesis of methamphetamine-BSA conjugate 28

[00112] The precursor derivative **27** was made in a similar manner to that for the amphetamine label analog as described in U.S. Patent 5,501,987 (Example 1), except that *d*-methamphetamine (free base) was used as the starting material, and the first step was performed with methylene chloride as the solvent.



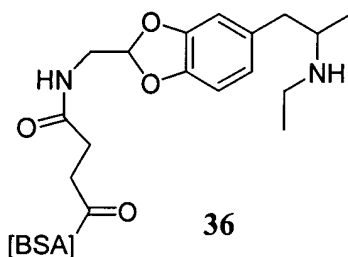
[00113] The methamphetamine-BSA conjugate **28** was then made by reaction of **27** with bovine serum albumin in a similar manner to that described above in Example 15, except that dialysis against 50 mM K₂CO₃ was performed without adjustment of the pH. Coomassie Blue protein assay on the final recovered retentate indicated the protein concentration to be 11.6 mg/mL.



Example 17. Synthesis of MDEA-BSA conjugate 36

[00114] MDEA-BSA conjugate **36** was synthesized as described in copending, commonly assigned U.S. Serial No. _____ filed contemporaneously herewith. A solution of 500 mg of bovine serum albumin (BSA) in 6.7 mL of 50 mM potassium phosphate (pH 7.5) was cooled in an ice-bath. To the solution was added 8.5 mL of DMSO dropwise, and the reaction mixture was maintained below room temperature. To the protein solution was added a solution of 12 mg (0.022 mmol) of N-(5-{2-[ethyl-(2,2,2-trifluoro-acetyl)-amino]-

propyl}-benzo[1,3]dioxol-2-ylmethyl)-succinamic acid 2,5-dioxo-pyrrolidin-1-yl ester (compound **2L** in U.S. Serial No. _____) in 1.5 mL of anhydrous DMF dropwise. The reaction mixture was allowed to stir at room temperature 48 hours. The resulting conjugate was placed in a dialysis tube (10,000 MW cut-off) and was dialyzed in 1 L of 70% DMSO in 50 mM potassium phosphate (pH 7.5, 3 changes, at least 3 hours each), 1 L of 50% DMSO in 50 mM potassium phosphate (at least 3 hours), 1 L of 30% DMSO in 50 mM potassium phosphate (at least 3 hours), 1 L of 10% DMSO in 50 mM potassium phosphate (at least 3 hours) at room temperature. The trifluoroacetamido group of the conjugate was deprotected by dialysis of the resulting conjugate against 10% ammonium hydroxide for 3 days (1 L each for approximately 24 hours each), followed by 6 changes with 50 mM potassium phosphate (pH 7.5) at 4°C (1 L each). The protein concentration was determined to be 7.12 mg/mL using BioRad Coomassie blue protein assay (Bradford, M., *Anal. Biochem.* 72:248,1976). A total of 45 mL of the conjugate was obtained.



Example 18. Development of hybridomas to ecstasy-class drugs using MDMA immunogen 12a

Immunizations

[00115] BALB/c female mice 18-24 weeks of age were immunized with **12** (T=KLH, **12a**). The immunogen was emulsified in Freund's adjuvant and administered via intraperitoneal (IP) injection. Injections were given at no less than 21-day intervals and typically comprised 50 µg of the conjugate in 100 µL of 50% saline/50% adjuvant emulsion. Complete Freund's adjuvant was used for the primary immunization and incomplete Freund's adjuvant used thereafter. A booster immunization of 50 µg in the same emulsion was administered IP 4 days prior to fusion.

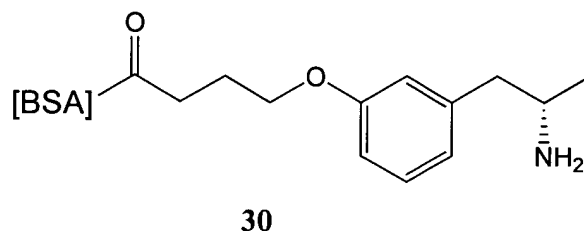
Fusion

[00116] On the day of performing the fusion, the mouse was killed by cervical dislocation and a blood sample taken. The spleen and popliteal, inguinal, subclavial, and deep inguinal lymph nodes were harvested and pooled. These were ground between two sterile glass slides to release the lymphocytes. One-half of the resulting lymphocyte suspension was used to fuse with the F0 myeloma cell line, and the remaining half was fused with the P3 myeloma. (Both myelomas were from ATCC.)

[00117] Fusion consisted of adding myeloma cells (1/5 the number of lymphocytes), washing via centrifugation, resuspension in serum-free warm Iscove's Modified Dulbecco's Media, and re-centrifugation. The centrifuge tubes containing the resulting pellets were gently tapped to loosen the cells, then 1 mL of warmed PEG/DMSO solution (Sigma Chemical Co.) was slowly added with gentle mixing. The cells were kept warm for 1.5 minutes, after which pre-warmed, serum-free IMDM was added at the following rates: 1 mL/min, 2 mL/min, 4 mL/min, and 10 mL/min. Then the tube was filled to 50 mL, sealed, and incubated for 15 minutes. The cell suspensions were centrifuged, the supernatant decanted, and IMDM containing 10% fetal calf serum was added. The cells were centrifuged once again and re-suspended in complete cloning medium. This comprised IMDM, 10% FCS, 10% Condimed H1 (Roche Molecular Systems, Pleasanton, CA), 4 mM glutamine, 50 μ M 2-mercaptoethanol, 40 μ M ethanolamine, and pen/strep antibiotics. The cells were suspended at a density of 4×10^5 lymphocytes/mL, distributed 100 μ L/well into sterile 96-well microculture plates, and incubated at 37°C in 5% carbon dioxide for 24 hours. The next day, 100 μ L of HMT selective medium (cloning medium + 1:25 HMT supplement from Sigma Chemical Co.) was added. On the 6th day of incubation, approximately 150 μ L of media was drawn from each well using a sterile 8-place manifold connected to a light vacuum source. One hundred fifty microliters of HT media was then added. This comprised cloning medium + 1:50 HT supplement (Sigma Chemicals). The plates were returned to the incubator and inspected daily for signs of growth. When growth was judged sufficient, wells were screened for antibody production via ELISA.

ELISA screening

[00118] Microplates were coated with 100 μ L methylenedioxymethamphetamine-BSA conjugate **12** (T=BSA, **12b**) at a concentration of 1 mg/mL, and separate microplates were coated with either 100 μ L methamphetamine-BSA (MAMP-BSA) **28** at a concentration of 1 mg/mL or with 100 μ L of amphetamine-BSA (AMP-BSA) **30** at a concentration of 1 mg/mL.



All dilutions were in 0.1 M carbonate buffer, pH 9.5.

[00119] The plates were incubated covered for 1 hour at 37°C (humidified). The plates were then emptied and filled with a post-coat solution consisting of Tris buffer, 1% gelatin hydrolysate, 2% sucrose, and 0.17% TWEEN 20. (All reagents were from Sigma Chemical Co.) The plates were incubated covered for an additional 1 hour at 37°C (humidified), after which they were washed with phosphate-buffered saline containing 0.1% TWEEN 20. The plates were then filled with a 2% sucrose solution in 0.15 M Tris, pH 7.2-7.4, briefly, then emptied and allowed to air dry at room temperature. When dried, the plates were packed in ZIPLOC (Dow Chemical Co.) plastic bags containing several desiccant pillows, sealed, and stored at 4°C until use.

Primary fusion screen

[00120] For the primary screening of the growing clones from the fusion plates, only the MDMA-BSA **12** (T=BSA, **12b**) coated plates were used. Fifty microliters of PBS was added to each well, followed by 50 μ L of the sample of culture media from wells on the fusion plate, diluted 1:10 in PBS. The plates were incubated covered for 1 hour at 37°C, then washed with PBS-TWEEN (0.1%). The wells were then filled with 100 μ L of goat

anti-mouse IgG-HRP conjugate (Zymed Labs), diluted in PBS-TWEEN, and the plates re-incubated for 1 hour. The plates were then washed again and 100 μ L of K-Blue Substrate (Neogen Corp.) added. This was allowed to develop for 5-15 minutes, the reaction being stopped by the addition of 100 μ L of 1 N HCl. Color was read by means of a microplate reader at 450 nm and collected by computer for analysis. Those wells that showed the presence of antibody binding to MDMA-BSA **12** (T=BSA, **12b**) were selected for further processing. Cells were subjected to limiting dilution subcloning, and upon appearance of growth, were tested by a secondary screen.

Secondary screen

[00121] Four plates coated with the MDMA-BSA conjugate **12** (T=BSA, **12b**) were prepared by adding 50 μ L of phosphate buffered saline (PBS) to the wells of one plate, 50 μ L of a solution of free MDMA (800 ng/mL) to the second plate, 50 μ L of a solution of MDEA (800 ng/mL) to the third plate, and 50 μ L of a solution of pseudoephedrine (8 μ g/mL) to the fourth plate. All drugs were dissolved in PBS. Fifty microliters of PBS were added to the wells of the MAMP-BSA (**28**) and AMP-BSA (**30**) coated plates.

[00122] When the growing subclones were judged ready for testing, 25 μ L of supernatant from the wells were taken and transferred to 96-well flexible plates. Culture medium was added to each well to provide a 1:10 dilution of the media sample. Fifty microliters of the diluted sample were transferred to each of the coated plates above. Subsequent processing was exactly as for the primary screen. Criteria for selection were binding to the MDMA-BSA conjugate **12** (T=BSA, **12b**), indication of inhibition by free MDMA and/or MDEA, and little or no inhibition by pseudoephedrine. Binding to the AMP-BSA (**30**) and MAMP-BSA (**28**) conjugates was for reference only.

[00123] Clones chosen were immediately subcloned and when ready, retested by the secondary screen procedure. Stable subclones were expanded, frozen, and the spent media used to determine specificity using the cross-reactivity assay.

Cross-reactivity assay

[00124] Supernatants were subjected to serial dilutions and re-tested in the ELISA screen above. The dilution providing for about a 50% reduction from the maximum OD was chosen for proceeding to cross-reactivity testing. This comprised repeating the preceding assay with the antibody at the chosen dilution and in the presence of varying concentrations of drugs.

Example 19. Development of hybridomas to ecstasy-class drugs using N-ethylamphetamine immunogen 26

Immunizations

[00125] Development of NEAMP 1.3: SJL female mice 18-24 weeks of age were immunized via a modified RIMMS method (Kilpatrick et al., *Hybridoma* 16:4, pp. 381-389, 1997). Immunogen 26 (Q=KLH, 26a) wherein Q is KLH was emulsified in incomplete Freund's adjuvant and administered via subcutaneous injection at 6 sites distributed over the nape of the neck and bilaterally to the calf and groin. Injections were given on day 0, day 3, day 6, and day 11. The respective dosages given were 50 µg, 25 µg, 12 µg, and 6 µg total amounts.

[00126] Development of NEAMP 48.2 and NEAMP 62.1: BALB/c female mice 18-24 weeks of age were immunized with immunogen 26 emulsified in complete Freund's adjuvant for the initial injection and in incomplete Freund's adjuvant for subsequent injections. The initial immunization was subcutaneous using 25 µg of immunogen. Thirty days later, the same dosage and route of injection was repeated. Ninety-seven days after the second immunization, a third was administered in which the dose was 100 µg via intraperitoneal injection. Approximately 11 months later, a final immunization the same dosage and route as the third was given, and the animal was used for fusion 4 days later.

Fusion

[00127] Four days subsequent to the final immunization, mice were killed via exsanguination. The popliteal, inguinal, subclavial, and deep inguinal lymph nodes were harvested and pooled. These nodes were ground between two sterile glass slides to release the lymphocytes. For development of NEAMP 1.3, one-half of the resulting lymphocyte suspension was used to fuse with the F0 myeloma cell line. The remaining half was fused with the P3 myeloma. (Both myelomas were obtained from ATCC.) For NEAMP 48.2 and NEAMP 62.1, only the F0 myeloma cell line was used.

[00128] Fusion consisted of adding myeloma cells (1/5 the number of lymphocytes), washing via centrifugation, resuspension in serum-free, warm Iscove's Modified Dulbecco's media, and re-centrifugation. The centrifuge tubes containing the resulting pellets were gently tapped to loosen the cells, then 1 mL of warmed PEG/DMSO solution (Sigma Chemical Co.) was slowly added with gentle mixing. The cells were kept warm for 1.5 minutes, after which pre-warmed, serum-free INMM was added at the following rates: 1 mL/min, 2 mL/min, 4 mL/min, and 10 mL/min. Then the tube was filled to 50 mL, sealed, and incubated for 15 minutes. The cell suspensions were centrifuged, the supernatant decanted, and IMDM containing 10% fetal calf serum was added. The cells were centrifuged once again and resuspended in complete cloning medium. This contained IMDM, 10% FCS, 10% Condimed H1 (Roche Molecular Systems, Pleasanton, CA), 4 mM glutamine, 50 μ M 2-mercaptoethanol, 40 μ M ethanolamine, and pen/strep antibiotics. The cells were suspended at a density of 4×10^5 lymphocytes/mL, distributed 100 μ L/ well into sterile 96-well microculture plates, and incubated at 37°C in 5% CO₂ for 24 hours. The next day, 100 μ L of HMT selective medium (cloning medium + 1:25 HMT supplement from Sigma Chemicals) was added. On the 6th day of incubation, approximately 150 μ L of media was drawn from each well using a sterile 8-place manifold connected to a light vacuum source. One hundred fifty microliters of HT media were then added. This comprised cloning medium + 1:50 HT supplement (Sigma Chemical Co.). The plates were returned to the incubator and inspected daily for signs of growth. When growth was judged sufficient, wells were screened for antibody production via ELISA.

ELISA screening

[00129] Microplates were coated with 100 μ L methamphetamine-BSA conjugate **28** (for screening for NEAMP 1.3) or 100 μ L of MDEA-BSA conjugate **36** (for screening for NEAMP 48.2 and NEAMP 62.1), and separate microplates were coated with 100 μ L N-ethylamphetamine-BSA **26** (Q=BSA, **26b**) at 1 μ g/mL in 0.1 M carbonate buffer, pH 9.5, for 1 hour at 37°C (humidified). The plates were then emptied and filled with a post-coat solution containing Tris buffer, 1% gelatin hydrolysate, 2% sucrose, and 0.17% TWEEN 20. (All reagents were from Sigma Chemical Co.) The plates were incubated for an additional 1 hour at 37°C (humidified), after which they were washed with phosphate-buffered saline containing 0.1% TWEEN 20. The plates were then filled with a 2% sucrose solution in 0.15 M Tris, pH 7.2-7.4, briefly, then emptied and allowed to air-dry at room temperature. When dried, the plates were packed in ZIPLOC bags containing several desiccant pillows, sealed, and stored at 4°C until use.

[00130] When the growing clones were judged ready for testing, 25 μ L of supernatant from the wells were taken and transferred to 96-well flexible plates. Culture medium was added to each well to provide a 1:10 dilution of the media sample. One hundred microliters of the diluted sample were transferred to each of the coated plates above. The plates were incubated covered for 1 hour at 37°C, then washed with PBS-TWEEN. The wells were then filled with 100 μ L of goat anti-mouse IgG-HRP conjugate (Zymed Labs) diluted in PBS-TWEEN and the plates re-incubated for 1 hour. The plates were then washed again, and 100 μ L of K Blue substrate (Neogen Corp) was added. This was allowed to develop for 5-15 minutes, the reaction being stopped by the addition of 100 μ L of 1 N HCl. Color was read by means of a microplate reader at 450 nm and collected by computer for analysis. A criterion for selection was binding to the methamphetamine-BSA conjugate **28** (for selection of NEAMP 1.3) or MDEA-BSA conjugate **36** (for screening for NEAMP 48.2 and NEAMP 62.1). Clones chosen were immediately subcloned and, when ready, retested. Stable subclones were expanded, frozen, and the spent media used to determine specificity using the cross-reactivity assay.

Cross-reactivity assay

[00131] Supernatants were subjected to serial dilutions and re-tested in the ELISA screen above. The dilution providing for about a 50% reduction from the maximum OD was chosen for proceeding to cross-reactivity testing. This comprised repeating the preceding assay with the antibody at the chosen dilution and in the presence of varying concentrations of drugs. The charts shown in Figures 4 and 5 present the results of such determination, while Table 2 (*vide supra*) shows the percentage cross-reactivity determined.

Example 20. ONLINE DAT II-type assay (KIMS) with antibodies and conjugates of the invention using HITACHI clinical chemistry analyzer

Preparation of NEAMP 48.2 microparticle reagent

[00132] 100 mL of 50 µg/mL NEAMP 48.2 antibody was incubated with 100 mL 1% 0.201 µm latex particles in 50 mM MES buffer, pH 6.5, overnight at room temperature. The particles were blocked with 10 mL of 100 mg/mL BSA in 50 mM MES buffer, pH 6.5, for 2 hours. Latex was washed with 50 mM MOPS buffer, pH 7.1, using tangential flow filtration system.

Conjugation of N-ethylamphetamine to aminodextran

[00133] Ninety mg of aminodextran (Ghoshal et al., Publ. No. US 2003/0060431 A1, page 12, paragraphs 147-152, March 27, 2003.) were dissolved in 5 mL DMSO. Ten mg of **24** were dissolved in 1 mL DMSO and added dropwise to the solution of the polymer carrier and stirred at room temperature overnight. The mixture was placed into 10,000 MW cut-off dialysis tubing (SNAKESKIN, Pierce) and dialyzed in 1 liter of 80% DMSO overnight at room temperature. This was then dialyzed using a step-down gradient in 1 liter of 60% DMSO, 1 liter of 40% DMSO, 1 liter of 20% DMSO, and 4 liters of deionized water, for at least 3 hours each at room temperature. This was then dialyzed with 1 liter of 100 mM K₂CO₃, adjusted to pH 13 with KOH, for 4 days with 4 changes of buffer. A final dialysis

step included 4 liters of deionized water for at least 2 days with 4 changes. The conjugate was then lyophilized.

Preparation of reagents

[00134] Reagent R2, microparticle reagent, pH 7.1, had the following composition:

antibody-coated microparticle	0.1% solids
3-morpholinopropanesulfonic acid	25 mM
3-morpholinopropanesulfonic acid, sodium salt	25 mM
BSA	0.1% (w/v)
sodium azide	0.09% (w/v)

[00135] Reagent R1, conjugate reagent, pH 7.1, had the following composition:

N-ethylamphetamine conjugate	0.125 µg/mL
piperazine-1,4-bis(2-ethanesulfonic acid) disodium salt	144 mM
piperazine-1,4-bis(2-ethanesulfonic acid)	31 mM
BSA	0.1% (w/v)
sodium azide	0.09% (w/v)
polyacrylic acid	1.5% (w/v)

Assay protocol

[00136] The assay was carried out using an HITACHI 917 analyzer (Roche Diagnostics). 10 µL of sample was pipetted into the cuvette and was immediately followed by the addition of 180 µL conjugate reagent (R1). The mixture was incubated for about 90 seconds, and 80 µL of microparticle reagent (R2) was added to the cuvette. The reaction mixture was incubated at 37°C for approximately 8 minutes, during which time the particle agglutination reaction was monitored at 505 nm. A standard curve was devised based on a 2-point end measurement after R2 addition.

Results

[00137] Cross-reactivity of NEAMP 48.2 and NEAMP 62.1 antibodies in an ONLINE DAT II-type assay (KIMS) is shown in Tables 3 and 4, respectively. Standard curves for NEAMP 48.2 and NEAMP 62.1 are shown in Figures 4 and 5.

[00138] The foregoing detailed description and examples have been provided by way of explanation and illustration and are not intended to limit the scope of the appended claims. Many variations in the presently preferred embodiments illustrated herein will be obvious to one of ordinary skill in the art and remain within the scope of the appended claims and their equivalents.